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# Evaluation of Genetically Engineered Herpes Simplex Viruses as Oncolytic Agents for Human Malignant Brain Tumors[1]

**Samita Andreansky, Liliana Soroceanu, Edward R. Flotte, Joany Chou, James M. Markert, G. Yancey Gillespie,[2] Bernard Roizman, and Richard J. Whitley**

*Brain Tumor Research Laboratories, Division of Neurosurgery, Department of Surgery [L. S., E.R. F., J. M. M., G. Y. G.], Division of Clinical Virology, Department of Pediatrics [S. A., R. J. W.], Departments of Microbiology [S. A., G. Y. G., R. J. W.] and Medicine [R. J. W.], University of Alabama at Birmingham School of Medicine, Birmingham, Alabama 35294-0006, and The Marjorie B. Kovler Virology Oncology Laboratory, University of Chicago, Chicago, Illinois 60630 [J. C., B. R.]*

## ABSTRACT

**Earlier studies have shown that genetically engineered herpes simplex viruses (*e.g.*, HSV-1) are effective in killing malignant tumor cells both *in vitro* and in various murine tumor models. This report focuses on a panel of five genetically engineered viral mutants of the $\gamma_1$*34.5* gene, which was shown previously to cause reduction in viral replication and associated neurovirulence of HSV. These include R3616, which has both copies of $\gamma_1$*34.5* deleted, R4009, which has a stop codon inserted after codon 28 in both copies of the $\gamma_1$*34.5* gene, R849, which contains a *lacZ* gene inserted in place of the $\gamma_1$*34.5*, R908, which lacks 41 codons in frame after codon 72 of the $\gamma_1$*34.5*, and R939, which carries a stop codon precluding the translation of the COOH-terminal domain of the $\gamma_1$*34.5* gene. We report the following: (*a*) all five mutant HSVs were avirulent in experimental animals but were cytotoxic for human tumor cells *in vitro* and *in vivo*; (*b*) the $\gamma_1$*34.5*$^-$ HSV replicated in human glioma cells almost as efficiently as wild-type HSV-1(F) based on replication assays, *in situ* hybridization for viral DNA, and expression of infected cell protein 27; (*c*) capacity of mutant HSVs to kill human cells derived from glioblastoma multiforme (CH-235MG, D-37MG, D-54MG, D-65MG, U-251MG, U-373MG, and SK-MG-1), anaplastic astrocytoma (Hs-683), anaplastic glioma (U-87MG and U-138MG), gliosarcoma (D-32GS), or normal human astrocytes demonstrated that glioma cells varied in their susceptibility to HSV-mediated cytotoxicity and that cultured astrocytes were two to three orders of magnitude less susceptible to killing than were malignant glia; and (*d*) *scid* mice, which received 0.5 or 5 × $10^6$ plaque-forming units of R4009, either were coinoculated at the time of intracranial transplantation with $10^6$ U251MG or D-54MG human glioma cells or received the cells intratumorally 5 days after tumor induction and experienced significant increases in median survivals, with no histopathological indication of an infectious encephalitic process. Genetically engineered $\gamma_1$*34.5*$^-$ HSV mutants appear to be a potentially safe biotherapeutic agent for experimental treatment of uniformly fatal malignant brain tumors.**

## INTRODUCTION

Primary malignant brain tumors affect only a small proportion of the population in the United States yet remain one of the most difficult therapeutic challenges. The median survival of optimally treated patients with GBM,[3] the most frequent of the malignant gliomas, remains at less than 1 year, a statistic that has not changed in the last 30 years despite impressive developments in neurosurgical, interventive neuro-oncological, radioneurosurgical, and neuroimaging techniques. Remarkable advances in the genetics and biology of malignant tumors, and malignant gliomas in particular, have contributed greatly to our understanding of tumor development and progression and have given rise to novel therapeutic approaches. Gene therapy has been one area of focus, using vectors, often viruses, to insert immune-stimulating or drug-susceptibility genes (1). Another approach uses a genetically engineered HSV type 1 (HSV-1), not as a vector to transfer genes, but as a direct tumor cell killer. The latter approach is particularly appealing because the capacity of genetically engineered HSVs to establish productive infection only in dividing tumor cells can be exploited as a means of selectively killing brain tumor cells without injuring adjacent normal brain tissue.

HSV-1 typically causes an initial mild to asymptomatic infection after transmission to cells of mucous membranes (reviewed in Ref. 2). HSV-1 genes are temporally expressed in a cascade of $\alpha$, $\beta$, and $\gamma$ genes. The $\gamma$ genes are further subdivided into $\gamma_1$ and $\gamma_2$ genes with $\gamma_1$ genes expressed earlier in infection. One of these $\gamma_1$ genes, $\gamma_1$34.5, is present in two copies and encodes a 263-amino acid protein. The 70-amino acid COOH-terminus is highly homologous to the mammalian growth arrest and DNA damage genes, *GADD34* (3, 4). Viral infection normally triggers a host stress response that shuts off protein synthesis and causes apoptosis, aborting viral replication (5). Analyses of the $\gamma_1$34.5 gene by mutagenesis have shown that it encodes two functions. One of these, which has been mapped in the 70-amino acid COOH terminus, blocks the shutoff of protein synthesis induced by the activated PKR kinase. Specifically, recent studies have shown that HSV-1 infection activates the PKR kinase. In the absence of a functional $\gamma_1$34.5 gene, the $\alpha$ subunit of the translation initiation factor eIF-$\alpha$2 is phosphorylated, resulting in total shutoff of protein synthesis (6). Mutations in the $\gamma_1$34.5 gene also render the virus aneurovirulent, although this may be due to an alternate function of this gene product. Various mutants of HSV-1 have been constructed to alter $\gamma_1$34.5 gene expression. These genetically engineered viruses have been proven to be highly attenuated in experimental animal systems with almost complete loss of neurovirulence (7, 8). Mutations of the $\gamma_1$34.5 gene include: deletion of both copies (R3616), insertion of a stop codon in the $NH_2$ terminus (codon 28) of both copies (R4009), substitution of $\gamma_1$34.5 with *LacZ*, the bacterial $\beta$-galactosidase gene (R849), a 41-codon deletion in the $NH_2$ terminus (R908), and insertions of stop codons in the COOH terminus (R939). With the exception of R849, construction of these viruses has been described elsewhere (4, 7). These engineered HSV-1 viruses are not capable of replication when injected at up to 1 × $10^6$ PFUs into the brains of normal mice.[4] However, these viruses can productively infect and kill actively dividing mouse or human glioma cells in culture (9, 10).

Recently, our laboratories (10) have shown that R3616 and R4009 HSV significantly prolonged survival of *scid* mice bearing intracranial

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[2] To whom requests for reprints should be addressed, at Brain Tumor Research Laboratories, THT 65, 1900 University Boulevard, University of Alabama at Birmingham, Birmingham, AL 35294-0006.

[3] The abbreviations used are: GBM, glioblastoma multiforme; HSV, herpes simplex virus; scid, severe combined immunodeficient; FBS, fetal bovine serum; tk, thymidine kinase; PFU, plaque-forming unit; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GCV, ganciclovir; ACV, acyclovir; PKR, protein kinase RNA-dependent; TEPSA, 3-aminopropyltriethoxysilane.

[4] E. Kern, unpublished data.

isografts of the spontaneous mouse glioma, MT539MG. Given that HSV-1 viruses can infect mouse glioma cells *in vitro* and produce a significant prolongation of survival of tumor-bearing mice, even when injected 4 days after tumor implantation, we hypothesized that these genetically engineered viruses should be able to kill human glioma cells implanted in this murine model and, therefore, may be useful as a biotherapeutic agent in treating malignant gliomas.

Herein, we describe the ability of five different genetically engineered HSV-1 $\gamma_1 34.5^-$ mutants to infect, replicate in, and kill different human glioma cell lines *in vitro*. One of these, R4009, was shown to prolong host survival and produce cures of human glioma cells transplanted into the brains of *scid* mice. Our observations that normal human astrocytes in culture require a 2–3 log higher multiplicity of infection with these genetically engineered HSV-1 to produce the same degree of *in vitro* cytotoxicity seen with malignant glia and that no evidence of encephalitis was detected in brains of *scid* mice injected intracranially suggest that these agents may eventually prove to be useful in therapy of intracranial neoplasms in humans.

## MATERIALS AND METHODS

**Cell Lines.** The following human cell lines were used: GBM-derived D-37MG, D-54MG, D-65MG, U-251MG, and U-373MG and gliosarcoma-derived D-32GS (all from Dr. D. D. Bigner, Duke University); neuroglial-derived SK-MG-1 (from G. Cairncross, London, Ontario, Canada); GBM-derived CH-235MG; and anaplastic glioma-derived Hs-683, U-87MG, and U-138MG (from American Type Culture Collection, Rockville, MD). Cells were maintained in DMEM mixed 1:1 with Ham's nutrient mixture F-12 (DMEM/F12), supplemented with 2 mM L-glutamine and 7% FBS. Cells were harvested from logarithmic-phase growth cultures by brief exposure to 0.5% trypsin and 0.53 mM EDTA (Life Technologies, Inc.). Vero cells (American Type Culture Collection) were used to grow the parental virus, HSV-1(F), or recombinant viruses in minimal essential medium supplemented with 10% heat-inactivated FBS and gentamicin (50 μg/ml).

**Normal Astrocyte Culture.** Normal human cerebral cortical tissue was obtained from patients undergoing surgical resection of epileptic foci as the standard treatment for intractable epilepsy. In accordance with Institutional Review Board guidelines, all procured tissues were released by attending neuropathologists as tissue in excess of that needed for diagnostic purposes. Brain tissue was transported in sterile tissue culture medium on ice to the laboratory and processed immediately for culture as follows. Blood clots and debris were removed aseptically, and the tissue was minced into 1–2-$mm^3$ fragments and disaggregated by sequential 20-min exposures to trypsin (0.5%) and EDTA (0.53 mM) in Dulbecco's PBS. Cells were pelleted through FBS (200 × *g* for 8 min), resuspended in DMEM/F12 + 20% FBS + 1× Mito-X (Collaborative Research) and EGF (10 ng/ml; Chemicon International, Inc., Temecula, CA), and cultured in 150-$cm^2$ flasks. Cultures became confluent after 3–4 weeks and were harvested with trypsin-EDTA. Cells to be assayed with genetically engineered HSV were plated as described for that assay. Simultaneously, a portion of each cell suspension was also plated at $10^4$ cells/well in DMEM/F12 (+ 7% FBS + L-glutamine; 300 μl/well) in eight-well culture chambers (Lab-Tek). After 24–48 h, these slide cultures were fixed in fresh 2% paraformaldehyde and stained by the avidin-biotin peroxidase method with a panel of monoclonal antibodies to determine the degree of cell type purity. Monoclonal antibodies used were: 4A11.H9 (anti-GFAP), 15E2E2 (anti-S100 protein; Ref. 11), 20F3 (anti-glioma-derived growth factor-2; Ref. 12), 12A12.D7 (anti-transforming growth factor-β; Ref. 12), 5C3.F8 (anti-vascular endothelial growth factor; Ref. 13), and smC1.2 (anti-insulin-like growth factor-I; Ref. 14).

**Viruses.** HSV-1(F) is the wild-type prototype virus from which all $\gamma_1 34.5^-$ mutant viruses were constructed (7). R3616 has 1000 bp deleted from the $\gamma_1 34.5$ gene between the *Bst*EII site at the 28th codon and *Stu*I site (Fig. 1). R4009 contains an in-frame translation termination at the *Bst*EII site. In the recombinant virus R849, the 1 kilobase pair deleted from R3616 was replaced with the *lacZ* gene inserted such that it was under the control of the $\gamma_1 34.5$ promoter. The virus was constructed by cotransfection of the intact DNA of virus R4002 with a plasmid in which the coding domain of the $\gamma_1 34.5$ gene was

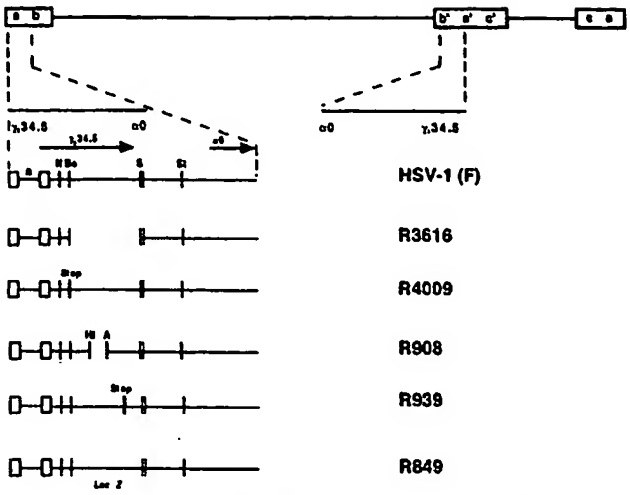


Fig. 1. Schematic diagram of $\gamma_1 34.5$ mutations engineered into the HSV-1(F) wild-type virus. *Top line*, unique long and unique short sequences of the HSV-1 genome flanked by the inverted repeats enclosed in the *boxes*. Expanded domains of these repeat regions indicate positions of $\gamma_1 34.5$ and *α0* genes. *Lines with arrows*, transcribed sequences and the direction of transcription. Restriction endonucleases are: *N*, *Nco*I; Be, *Bst*EII; S, *Sac*I; St, *Stu*I; Hf, *Hin*fI; and A, *Ava*II.

replaced by the *lacZ* gene. R4002 contains the HSV-1 *tk* gene inserted in the 5'-terminus of the $\gamma_1 34.5$ gene as described elsewhere (7). The progeny of transfection were plated on 143TK$^-$ cells in the presence of bromouracildeoxyriboside. This procedure selects *tk*$^-$ viruses and because the *tk* gene is present in both copies of the $\gamma_1 34.5$ gene, the selected progeny of the transfection could be expected to contain deletions in both copies. Plaques growing under bromouracildeoxyriboside were screened for the presence of *lacZ* in both copies of the inverted repeats. Lastly, the *tk* gene was repaired to yield R849. Recombinant viruses R908 and R939 are described elsewhere (3). HSV R908 has a 41-codon deletion in frame after codon 72. R939 contains stop codons at the COOH terminus in all six reading frames inserted at codon 11 after the last three amino acid repeats of the $\gamma_1 34.5$ gene. All mutations were present in both copies of the $\gamma_1 34.5$ gene. In some studies, viruses in which the specific mutations had been repaired [R3616(R), R939(R), and R908(R)] were used to demonstrate the restoration of wild type-like virulence and thus confirm that any changes in phenotype of the mutant were, in fact, due to mutation engineered into the virus.

**Replication of $\gamma_1 34.5$ Mutants.** Replicate cultures of human glioma cell lines U251MG and D54MG seeded to subconfluency were either mock infected or exposed for 1 h at 37°C to 0.1 or 1 PFU of mutant viruses or parental HSV-1(F) per cell, then rinsed, overlaid with medium, and further incubated (37°C, 5% $CO_2$). Cell monolayers were harvested at 12, 24, 48, and 72 h after infection and sonicated; lysates were titered for infectivity on Vero cells monolayer cultures. At 48 h after infection, Vero cells were fixed and stained with May-Grünwald (Aldrich Chemical Company, Milwaukee, WI) and Giemsa (Sigma Diagnostics, St. Louis, MO) stains, and plaques were counted by light microscopy (15).

***In Situ* DNA Hybridization for Synthesis of Viral DNA in Human Glioma Cells.** U251MG and D54MG cells grown in four-chamber glass slides were infected with R3616, R4009, or HSV-1(F). Eighteen to 24 hr after infection, cells were fixed in acetone for 10 min and processed for *in situ* DNA hybridization using ENZO Color Gene DNA hybridization test kit (ENZO Diagnostics, New York, NY). Briefly, biotinylated HSV DNA probe was added to fixed cells and heated on a 92°C heating block for 2 min. Slides were removed from the heating block, and the reaction was allowed to proceed further for another 20 min at room temperature. The cells were next treated with posthybridization buffer for 10 min, washed, and then incubated with avidin-biotinylated horseradish peroxidase. Color development was achieved with 3-amino-9-ethylcarbazole mixed with hydrogen peroxide (15 min at room temperature). Cells were washed and counterstained with methyl green for 30 s.

**Immunoblot Analyses of Viral Proteins.** U251MG and D54MG cells were infected with recombinant $\gamma_1 34.5$ mutants or HSV-1(F) (positive con-

trol). At specified times after infection, cell lysates were harvested, sonicated, solubilized in buffer containing SDS, and subjected to electrophoresis in denaturing polyacrylamide gels. Reagents for PAGE were purchased from Boehringer Mannheim (Indianapolis, IN). Resolved proteins were subsequently transferred to nitrocellulose for immunoblotting (16). Briefly, nitrocellulose blots were reacted with mouse monoclonal antibody to infected cell protein 27 (ICP27), the product of the $\alpha 27$ gene (Advanced Biotechnology, Inc., Columbia, MD) at a 1:1000 dilution (overnight at 4°C), rinsed, and further incubated (30 min at room temperature) with rabbit antimouse IgG (Organon Teknika, West Chester, PA). Bound secondary antibody was detected with $^{125}$I-labeled protein A (1 h at room temperature) and visualized by autoradiography. Protein A from *Staphylococcus aureus* (Pharmacia Laboratories, Uppsala, Sweden) was radioiodinated using Na$^{125}$I (15.5 mCi/μg of sodium iodide; Amersham Corp., Arlington Heights, IL) according to the method of Greenwood *et al.* (17).

For immunocytochemistry, glioma cells were seeded into eight-chamber slide trays (Nunc, Inc., Naperville, IL), infected with the indicated HSV-1 mutants, and 24–48 h later, fixed with 2% paraformaldehyde. Cells were permeabilized in methanol for 30 s, rehydrated, and incubated (overnight at 4°C) with either monoclonal anti-ICP27 (Advanced Biotechnologies, Inc.) or polyclonal anti-HSV (Dako). Slides were washed and incubated with the relevant biotinylated anti-globulins (Vector Laboratories, Burlingame, CA). Detection of secondary antibody was performed with the avidin-biotin method, with production of a colored substrate using 10 mg of 3,3′-diaminobenzidine (Sigma) in Tris-HCl (pH 7.3) with 0.006% $H_2O_2$. Nickel sulfate (0.8%) was added to some substrate solutions to produce a blue-black instead of a brown reaction product. Slides were counterstained with Mayer's hematoxylin, dehydrated in graded ethanols and then xylenes, and mounted with Accu-mount media (Baxter Scientific Products, Inc.).

***In Vitro* Cytotoxicity Assays.** Two cytotoxicity assays were used. In the first, cells were visually inspected for evidence of cytotoxicity, and in the second, cellular cytotoxicity was measured by the alamarBlue assay. Cells were plated at 4–10 × 10$^3$ cells/well in duplicate 96-well microplates in DMEM/F12 (+ 7% FBS + L-glutamine; 90 μl/well) and incubated 24 h (37°C at 5%$CO_2$). Virus was then added in four replicate wells each at ratios of 0.1, 0.33, 1.0, 3.3, 10, 33, and 100 PFU/seeded cell. Saline was added to four replicate wells per cell line in place of virus as a control for estimation of maximal cell viability. alamarBlue dye (Alamar Biosciences; 40 μl/well) was added 3 days after virus infection, and the microplates were incubated (3 h at 37°C). The alamarBlue assay measures altered mitochondrial function, an indicator of cell metabolism. Results are expressed as $A_{562\,nm}$ corrected by subtraction of $A_{590\,nm}$ using an automated plate reader (EL310; BioTek, Inc., Winooski, VT). All experiments with mutant viruses were repeated a minimum of three times; the cytotoxicity of HSV-1(F) was determined in duplicate experiments for comparison purposes for some of the cell lines. The percentage of reductions in dye conversion produced by virus when compared to maximal mitochondrial function (100%) were plotted as a function of the quantity of virus measured by PFU to which the cells were exposed, and the number of viral PFUs required to produce a 50% reduction was designated as the 50% toxic dose, hereafter defined as the PFU/TD$_{50}$. To determine the optimum number of cells required to generate a sufficiently dynamic range in which to measure cytolytic changes in virus-infected cultures, several different cell types were plated under the same conditions, with the number of cells per well varying from 4 to 20 × 10$^3$/well, and cell viability was assessed at different time points with alamarBlue. As an example, 8 × 10$^3$ cells/well proved to be optimal for detection of altered mitochondrial function of the neuroblastoma-derived cell lines, whereas 4 × 10$^3$ cells/well were used for the faster-growing glioma cell lines. As reported previously (13), this assay demonstrated a direct, linear correlation between cell number and dye conversion that was cell line specific.

**Acyclovir Sensitivity.** Subconfluent monolayers of U251MG and D54MG cells grown in multiwell plates were exposed to one PFU of R3616, R4009, or HSV-1(F) per cell. After adsorption (1 h at 37°C), cells were washed and treated with a range of concentrations (0.5–10 μg/ml) of acyclovir (Burroughs-Wellcome Company, Research Triangle Park, NC) purchased from the University of Alabama Hospital pharmacy. One set of cells served as the untreated control. Supernatant fluids and cell monolayers were harvested 24 h after infection, and plaque formation was quantified on Vero cells as described above.

**Intracranial Glioma Model.** The ability of R4009 to cause regression of human glioma cells xenotransplanted into the right cerebral hemisphere was tested in C.B-17 *scid* mice. In one assay, groups of 10 mice each received 10$^6$ D-54MG human glioma cells concurrently with one of two different doses of virus in a total of 5 μl (Winn-type assay). Alternatively, in delayed-therapy experiments, glioma cells were injected in 5 μl, and different virus doses were injected (5 μl) intratumorally 5–7 days later. Corresponding control groups of 10 mice each received excipient solution (serum-free DMEM/F12 + 5% methyl cellulose) in place of virus. All mice were anesthetized by i.p. administration [ketamine (20 mg/ml) plus xylazine (0.3 mg/ml) in saline at 0.07 ml/10 g of body weight]. A midline scalp incision was made, and a 0.5-mm burr hole was made 1.5–2.0 mm to the right of the midline and 0.5–1.0 mm posterior to the coronal suture. Tumor cells (with or without virus) were resuspended in excipient solution and stereotactically injected using a 250-μl Hamilton syringe with a 30-gauge needle mounted in a Stoelting stereotaxic apparatus. The needle was inserted vertically through the burr hole to a depth of 2.5 mm. Forty-five to 60 s after injection, the needle was slowly withdrawn, and the incision was closed with 9-mm Michel wound clips. Mice were returned to sterile microisolator polycarbonate cages, placed over a heating pad until recovery, and provided autoclaved lab chow and sterile water *ad libitum*. As mice became moribund from progressive tumor growth, they were euthanized, and their survival time was taken as the date of euthanasia. Determination of this end point was based on the absence of the characteristic avoidance behavior when touched. Animal studies were conducted in accordance with guidelines for animal use and care established by The University of Alabama at Birmingham Animal Resource Program and the Institutional Animal Use Review Board.

**Histochemical/Cytochemical Studies.** For demonstration of viral replication in gliomas *in vivo*, mice were inoculated with D-54MG cells intracranially and 5 days later received 5 × 10$^6$ PFUs of R849. Three to 5 days later, mice were anesthetized, perfused intracardially with PBS followed by 4% paraformaldehdye in 12% sucrose. Brains were fixed an additional 24 h at 4°C and then sectioned coronally free-hand through the injection site. Brain slices were incubated overnight in X-gal solution [0.2% X-gal, 10 mM sodium phosphate (pH 7.0), 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6{\cdot}3H_2O$, and 3.3 mM $K_3Fe(CN)_6$] according to manufacturer's directions (Promega Corp., Madison, WI). Stained slices were embedded in OCT (Miles, Inc., Naperville, IL), frozen, and sectioned at 8–10 μm on a cryotome (Zeiss HM550Z); sections were mounted on TEPSA-coated slides (Aldrich Chemical Company, Milwaukee, WI). Sections were counterstained with Nuclear Fast Red (American Histology Reagent Company, Lodi, CA).

Moribund mice were killed, and their brains were immediately harvested for histopathological examination of the presence and extent of engrafted tumor. Tumor sections were stained with mouse monoclonal antibody to α HSV glycoprotein ICP27 (Advanced Biotechnologies, Inc., Columbia, MD). Portions of tumor and normal brain tissues were frozen for subsequent plaque assays to determine whether infectious virus particles could be recovered from these tissues.

**Replication of R4009 and HSV-1(F) in an Intracranial Mouse Glioma Model.** Replication of R4009 and HSV-1(F) was demonstrated in mice bearing U251MG gliomas by intratumoral injection of either 5 × 10$^6$ PFUs of R4009 or 10$^6$ PFUs of HSV-1(F) 5 days after tumor induction. One to three mice from each group were sacrificed at 3, 7, or 11 (R4009 only) days after infection, and brain tissues were harvested, homogenized, and assayed on Vero cells to determine the quantity of the infectious virus as described above. Mice receiving wild-type virus did not live beyond 6 days, and their brains were studied at that time.

**Statistical Methods.** *In vivo* survival data were converted to Kaplan-Meier plots, and the significance of the differences in median survival times for the various groups were calculated using log-rank statistical analysis routines (SURVCALC; Wiley-Addison Co., Sussex, England). Survival of all mice was followed up to 60 days. Mice surviving to this end point were killed, and their brains were fixed for histopathological examination for evidence of tumor presence and of virus effects.

## RESULTS

**Growth Characteristics of $\gamma_1 34.5$ Genetically Engineered HSV.** Critical to the selection of potential viral candidates for brain tumor therapy is their ability to replicate in human glioma cells. To determine whether $\gamma_1 34.5^-$ mutants were able to replicate in and kill

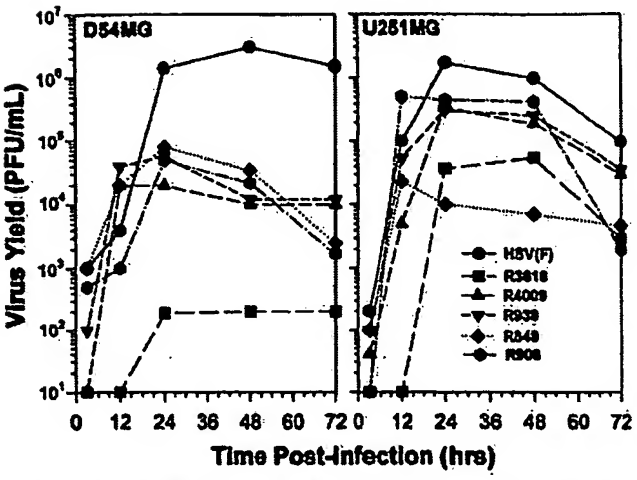


Fig. 2. Growth kinetics of HSV-1(F) and $\gamma_1 34.5$ recombinants in D-54MG and U-251MG human glioma cells exposed to 1 PFU/cell, demonstrating increasing viral replication of the five $\gamma_1 34.5$-defective HSV-1 mutants that was slightly less than that of wild-type HSV-1(F). Graphs shown are representative of results obtained from repeated assays.

human glioma cells, U251MG or D54MG cells were mock infected or exposed to 0.1 or 1 PFU of one of the recombinant viruses per cell. At specific intervals after infection, cell monolayers were harvested, and virus was titered using Vero cells. These studies were performed at least three times at both virus doses. Representative results with 1.0 PFU/cell indicate that all $\gamma_1 34.5^-$ recombinants were able to replicate in both human glioma cell lines with $10^2$-$10^3$-fold increases during the time period (24–72 h) in which these glioma cells normally experience exponential growth (Fig. 2). Titers of the recombinants and the parental virus HSV-1(F) peaked at 24 to 48 h after infection in both human glioma cell lines. However, in all cases, the titers of the mutant viruses lagged slightly below the wild-type HSV-1(F). R908 replicated to an average titer of $10^5$ PFUs/ml, which was closest to that obtained with the parental virus, HSV-1(F), $10^6$ PFUs/ml. Replication of the remaining mutants was less competent in both glioma cell lines; R3616 was the least efficient.

**Expression of Virus-specific Proteins in Human Glioma Cells.** To determine whether the $\gamma_1 34.5^-$ mutants were capable of expressing viral gene products in human glioma cells, electrophoretically resolved lysates of infected cells were transferred to nitrocellulose sheets and probed with antibody to ICP27. Results (Fig. 3) demonstrated that comparable amounts of ICP27 were expressed in D54MG cells infected either with R3616, R4009, R908, R939, or R849 at 5 or 12 h after infection when compared to that seen in glioma cells infected with wild-type or restored viruses [R3616(R), R908(R), and R939(R)].

**Synthesis of Viral DNA in Human Glioma Cells.** HSV α genes are expressed prior to synthesis of any viral DNA. The results described above do not indicate whether the viral replication machinery in human glioma cells infected with the $\gamma_1 34.5^-$ recombinants was activated efficiently when compared to the parental virus. An *in situ* hybridization assay for HSV DNA was performed with a biotinylated genomic DNA probe. R3616 and R4009 were chosen as representative viruses to infect either D54MG or U-251MG cells. At 12–14 h after infection, R3616 or R4009 were able to replicate viral DNA similar to that seen with wild-type (F) (data not shown). Hybridization with the HSV probe was not observed in the mock-infected cell cultures (data not shown). These data are consistent with previous reports that viral DNA synthesis is present in Vero cells infected with $\gamma_1 34.5$ mutants (3–6).

**Viral Cytotoxicity of Glioma Cell Lines.** The capacity of $\gamma_1 34.5$ mutants to produce a direct cytotoxic effect on malignant cells was also considered to be a desirable property for brain tumor therapy. Both human glioma cell lines initially tested proved to be susceptible to the cytopathic effect of the recombinant viruses. Cells became round, lost normal morphological features, and detached from the culture plate. As a quantitative measure of viral cytotoxicity, we used the alamarBlue assay. The number of PFUs of R4009 per target cell needed to produce a 50% decrease in dye conversion ($TD_{50}$) was generally consistent from assay to assay (the coefficient of variation ranged from 0.01 to 0.13) with each of the cell lines, permitting averaging of calculated PFU/$TD_{50}$ values. All 11 human glioma cell lines were sensitive to the cytotoxic effects of R4009, with PFU/$TD_{50}$ values that ranged from 1.4 to 13.6 at 3 days after addition of the virus (Table 1). When the abilities of the other four genetically engineered HSVs to kill D-54MG and U-251MG cells in culture were compared (Table 2), PFU/$TD_{50}$ values ranged from 1.4 to 14.3. U-251MG was slightly more sensitive to all viruses than D-54MG. Cytotoxicity of HSV-1(F) for those cell lines against which it was tested was comparable to that seen with the replication-competent mutants.

**Viral Cytotoxicity of Normal Astrocytes.** The astrocyte cultures used in these studies were 97–99% glial fibrillary acidic protein+, S100+, and transforming growth factor β+ but were negative for the other tested markers. The ability to kill normal human astrocytes *in vitro* was determined for HSV-1(F), R3616, R849, R4009, and R8306 (Table 3). As might be expected, the wild-type virus was as cytotoxic for astrocytes as it was for tumor cells. In contrast, R3616 was by far the least toxic to human astrocytes, with no discernible ability to kill astrocytes in culture (estimated PFU/$TD_{50}$, >1000). HSV R4009 was also minimally toxic (average PFU/$TD_{50}$, >1000) but did show some cell killing at later times. R849 was the most virulent virus for normal astrocytes, but it still required virus doses at least 2 logs above that needed to kill glioma cells ($TD_{50}$, >820 PFUs) effectively at 3 days. Because astrocytes proliferate slowly compared with glioma cells, we also examined metabolically active cells 6 days after viral infection.

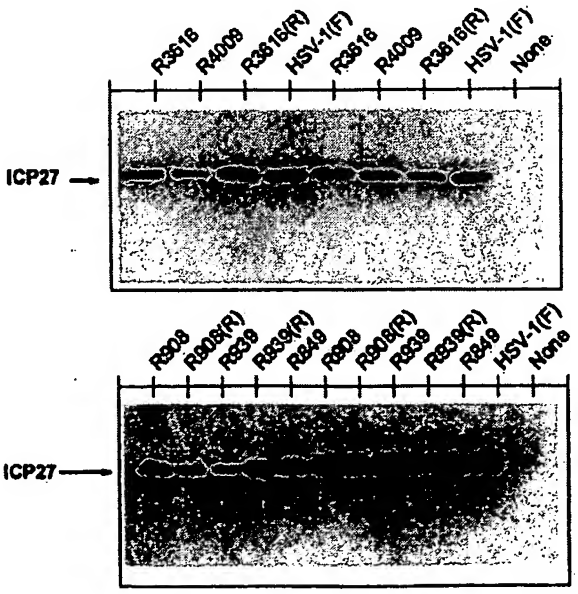


Fig. 3. Western blots demonstrating expression of ICP27 in D54MG cells infected with HSV-1(F) as well as five of the $\gamma_1 34.5^-$ HSV-1 mutants and three of the repaired viruses. In the *top panel*, SDS-PAGE resolved lysates from D54MG cells infected for 5 or 12 h with R3616, R4009, repaired R3616, or HSV-1(F) were probed with anti-ICP27. In the *bottom panel*, lysates from D54MG cells infected for 5 or 12 h with R908, repaired R908, R939, repaired R939, R849, or HSV-1(F) show abundant ICP27 by Western blot analysis.

Table 1 *Virulence of R4009 or HSV-1(F) in various human glioma cell lines*

| Cell line designation | Tumor of origin | R4009 PFU/$TD_{50}$[a] (mean ± SD) | HSV-1(F) PFU/$TD_{50}$[a] (mean) |
|---|---|---|---|
| CH-235MG | GBM | 7.4 ± 1.1 | ND |
| D-37MG | GBM | 12.6 ± 2.7 | ND |
| D-54MG | GBM | 4.3 ± 2.2 | 13.7 |
| D-65MG | GBM | 1.9 ± 1.2 | 1.2 |
| U-251MG | GBM | 3.4 ± 1.2 | 3.3 |
| U-373MG | GBM | 1.4 ± 0.9 | 1.4 |
| Hs-683 | Anaplastic astrocytoma | 2.2 ± 0.7 | ND |
| U-87MG | Anaplastic astrocytoma | 4.4 ± 0.4 | 0.9 |
| U-138MG | Anaplastic astrocytoma | 13.6 ± 5.2 | ND |
| SK-MG-1 | Anaplastic astrocytoma | 1.5 ± 0.7 | 1.2 |
| D-32GS | Gliosarcoma | 3.0 ± 1.0 | ND |

[a] Values for R4009 were obtained 3 days after virus infection and are means and SD of at least three separate experiments. Values from HSV-1(F) are means of two experiments. ND, not all cell lines were examined for sensitivity to the wild-type virus.

Table 2 *Comparative virulence of wild-type and genetically engineered HSV in D54MG and U-251MG human glioma cells*

| HSV Virus | D-54MG PFU/$TD_{50}$[a] | U-251MG PFU/$TD_{50}$ |
|---|---|---|
| R849 | 4.0 ± 1.9 | 2.43 ± 0.7 |
| R908 | 3.13 ± 0.6 | 1.36 ± 0.5 |
| R939 | 5.67 ± 0.9 | 5.14 ± 2.5 |
| R3616 | 14.3 ± 2.0 | 6.76 ± 1.8 |
| HSV-1(F) | 13.7[b] | 3.3 |

[a] Values were obtained 3 days after virus infections and are expressed as means and SD of at least three separate experiments.
[b] Values for wild-type HSV-1(F) are averages of two experiments.

Significantly lower PFU/$TD_{50}$ values were obtained for all except R3616, which continued to demonstrate poor cytotoxic activity for astrocytes.

**Acyclovir Sensitivity of $\gamma_1$34.5 Recombinant HSV-1.** One advantage of using $\gamma_1$34.5-defective HSV constructs is that they retain the *tk* gene, which makes them susceptible to such antiviral drugs as GCV and ACV. The susceptibility of recombinant viruses to these drugs provides a measure of safety by limiting viral persistence. We sought to demonstrate the extent to which these HSV constructs were sensitive to antiviral drugs using a plaque reduction assay. U251MG or D54MG cells were seeded, infected at 1 PFU/cell, and treated with 0.5, 0.7, or 1.0 μg of ACV/ml. At 24 h after infection, virus was harvested, and ACV sensitivity of R3616 and R4009 was compared with that of parental strain (F) by plaque assay. Our results indicated that R3616 and R4009 exhibited an $EC_{50}$ of 0.5 μg/ml (Fig. 4), which was consistent with the susceptibility of HSV-1(F) virus to ACV. Such sensitivity provides a measure of safety in the unanticipated instance of viral encephalitis.

**Demonstration of HSV Infection in Intracranial Gliomas.** As an initial step in determining whether $\gamma_1$34.5-defective HSV actually infect human glioma cells growing in mouse brain, we inoculated *scid* mice with $10^6$ D54MG glioma cells and, 5–7 days later, stereotactically injected R849 (5 μl) into the same injection site. Three days later, the animals were killed, and the distribution of infectious virus was ascertained by staining fixed brains in X-gal for evidence of β-galactosidase (*lacZ*) expression. Distribution of blue-staining tissue consistent with viral infection could be easily visualized grossly in free-hand coronal sections parallel through the needle track (Fig. 5, *top panel*). Microscopic examination of coronal sections of these brains revealed large, blue-stained tumor cells within the tumor bed, which tracked across the corpus callosum from the right to left hemisphere (Fig. 5, *middle* and *bottom panels*).

Next, we determined whether R4009 replicated in U251MG glioma cells implanted in the *scid* mouse brains. Glioma-bearing mice were injected intratumorally 5 days after tumor induction; HSV-1(F) was used as the positive control. At indicated intervals, brains were harvested, and virus titers were determined individually (Table 4). Virus was detected 3 days after inoculation, with peak virus recovery occurring approximately 7 days for mice receiving $10^5$ or $10^7$ PFUs of R4009. The quantity of virus recovered from brain tumors injected with the lower dose of HSV-1(F) virus was much higher than with R4009. Furthermore, none of the animals injected with HSV-1(F) virus survived beyond 6 days, even with the low viral dose. This further suggests that R4009 may be safe enough to be evaluated further as a potential candidate for brain tumor therapy.

***In Vivo* Effects of HSV-1 Mutant Viruses on Intracranial Glioma Xenografts.** Untreated *scid* mice injected intracranially with $10^6$ U251MG glioma cells became moribund in a predictable and uniform fashion with median survivals ranging from 32 to 35 days after tumor induction (average, 33.8 days). Mice receiving D54MG glioma cells intracranially became moribund at a significantly more rapid rate (median survival, 19 to 22 days; average, 20.4 days).

Using the Winn-type assay, R4009 used at 1 or 10 × $10^6$ PFUs/dose produced a significant ($P < 0.01$) increase in median survival, with 20 and 30% of the U251MG-bearing mice surviving to 75 days (Fig. 6*A*), at which point these long-term survivors were euthanized, and their brains were analyzed for tumor and infectious virus. Routine H&E histological examination of brains of moribund mice revealed large, space-occupying tumors. Survivors killed at 75–80 days had no discernible tumors, although areas of gliosis and calcification were occasionally seen. Surprisingly, portions of these brains from three of five long-term survivors that were processed for residual HSV contained $10^2$-$10^5$ PFUs/mg of tissue.

In the delayed therapy experiments, mice that had U251MG glio-

Table 3 *HSV virulence in normal human cultured astrocytes*

| HSV isolate | Assay day | NBO-874835 PFU/$TD_{50}$[a] | NBO-1808094 PFU/$TD_{50}$ | NBO-1299470 | NBO-1433672 |
|---|---|---|---|---|---|
| R849 | 3 | 473.0 | $>10^3$ | | |
| | 6 | 26.1 | 14.2 | | |
| R3616 | 3 | None | $>10^3$ | | |
| | 6 | None | $>10^3$ | | |
| R4009 | 3 | $>10^3$ | $>10^3$ | | |
| | 6 | 44.6 | 105.2 | | |
| HSV-1(F) | 3 | | | 15.7 | 4.9 |
| | 6 | | | 0.9 | 0.3 |

[a] Values are averages of at least three separate experiments with mutant viruses and duplicate experiments with HSV-1(F).

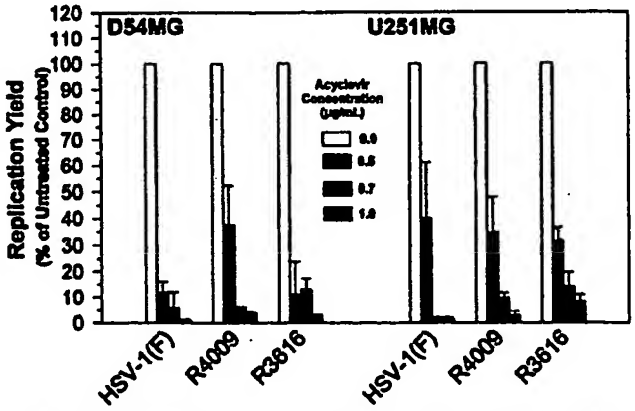


Fig. 4. Effect of graded doses of ACV on replication of HSV-1(F), R3616, and R4009 in D-54MG or U251MG human glioma cells. Intracellular virus was quantified by plaque assay on Vero indicator cells. Titers obtained for virus recovered from cultures maintained in the absence of ACV were designated as the maximum (100% of untreated controls) for each cell line. Viral titers in acyclovir-treated cultures were then expressed as a percentage of the maximum for each. *Bars*, SD.


Fig. 5. *A*, coronal sections of *scid* mouse brains after intracranial D54MG tumors were injected with $10^7$ PFUs of R849 and stained 3 days later with X-gal. Strong blue stain is grossly evident in lateral areas occupied by D54MG tumor cells. *B*, tissue sections stained with X-gal were counterstained with Nuclear Fast Red; the area adjacent to the needle track (*top*) revealed many large tumor cells (*arrows*) containing a blue, granular staining pattern; *bar*, 100 μm. *C*, section of corpus callosum (cc) in which large tumor cells, stained blue in a granular, cytoplasmic pattern, can be seen (*arrows*) moving away from the margin of tumor mass (*T*) at the *lower left*.

mas induced 5 days earlier were treated by intratumoral injection of 1 or 10 × $10^6$ PFUs of R4009 (Fig. 6*B*). HSV-treated mice also experienced significant survival, with median survival times of 48 and 49 days, respectively, compared with control animals (33 days). Again, we observed 25% of these tumor-bearers to be long-term survivors, and with the exception of one animal, these survivors appeared healthy. Surviving mice were killed at day 80 to harvest their brains for histological and virological studies. Low levels of R4009 HSV (<$10^2$ PFU/g) were detected in the rostral half of the brains sectioned coronally at the injection site, but there were no microscopically discernible changes in the H&E-stained caudal portions to suggest viral-mediated histopathology (data not shown).

In the Winn-type assay with mice bearing the more rapidly lethal D54MG gliomas, R4009 produced a significant prolongation in survival at both doses of virus injected, 0.5 and 5.0 × $10^6$ PFUs (Fig. 7*A*). All of the mice implanted with $10^6$ D54MG glioma cells comixed with 0.5 × $10^6$ PFU R4009 were long-term survivors, whereas control mice that received $10^6$ D54MG glioma cells mixed with an equivalent volume of saline all died, with a median survival of 18.5 days. Forty % of the mice that received D54MG cells mixed with 5.0 × $10^6$ PFUs R4009 prior to inoculation were long-term survivors; none of these animals died until after the last untreated control mouse had died. Inspection of the brain tissues of mice that died revealed that tumor was uniformly the cause of death in the 5 × $10^6$ PFUs group and that there was gliosis but no evidence of tumor in the brains of surviving mice of both groups that were killed. Because the D54MG glioma cells tended to kill the mice at a much more rapid pace than U251MG glioma cells, we increased the doses of R4009 virus administered 5 days after induction of D54MG gliomas. Although these mice survived significantly longer ($P < 0.015$) than the mock-treated (saline) mice, the 2-fold higher dose did not improve the number of long-term survivors (only 10%) in either group with HSV-treated tumors. The increase in survival for both treated groups was highly significant (Fig. 7*B*) compared with saline controls.

## DISCUSSION

Studies on the susceptibility of human glioma cells to genetically engineered $\gamma_1 34.5^-$ mutants suggested that: (*a*) human tumor cells are effectively killed within a 3-day period by *in vitro* exposure to doses of <15 PFUs/cell; (*b*) one mutant, R4009, was effective *in vivo* in prolonging median survivals of *scid* mice bearing intracranial human gliomas, in some instances curing these mice. However, it was also more virulent than other mutants, possibly because of low level of expression of stop codon inserted in frame into the $\gamma_1 34.5$ gene; and (*c*) most $\gamma_1 34.5^-$ HSV could be injected intracerebrally in *scid* mice at relatively high doses ($10^7$ PFUs) without inducing a fatal encephalitis.

There have been two major approaches to using viruses to treat brain tumors. The first is to use a replication-incompetent viral vector, such as retroviruses or adenoviruses, to shuttle antitumor genes, such as the HSV-1 *tk* (HSV-*tk*) gene, into the tumor cells. The oncolytic effect is not produced by the virus but by administrating a relatively nontoxic prodrug, which is converted into a toxic metabolite, poison-

Table 4 *Replication of R4009 and HSV-1(F) in intracranial gliomas in scid mice*

| Virus (PFU) | Days after virus injection | No. positive/no. injected | Virus titers (PFU/ml) |
|---|---|---|---|
| HSV-1(F) ($10^5$ PFU) | 3 | 3/3 | 1 × $10^5$ |
| | | | 2 × $10^6$ |
| | | | 1 × $10^7$ |
| | 6[a] | 1/1 | 1 × $10^5$ |
| R4009 ($10^6$ PFU) | 3 | 1/1 | 1 × $10^4$ |
| | 7 | 2/2 | 1 × $10^3$ |
| | | | 2 × $10^3$ |
| | 11 | 2/2 | 5 × $10^3$ |
| | | | 4 × $10^3$ |
| R4009 ($10^7$ PFU) | 3 | 1/1 | 1 × $10^4$ |
| | 7 | 2/2 | 1 × $10^3$ |
| | | | 1 × $10^6$ |
| | 11 | 2/2 | 1 × $10^4$ |
| | | | 1 × $10^3$ |
| Control (No virus) | 3 | 0/1 | 0 |
| | 7 | 0/2 | 0 |
| | 11 | 0/2 | 0 |

[a] No HSV-1(F) survivors after day 6.

ing the infected cell and, to some extent, adjacent cells by a "bystander effect." Death of the infected cell limits and terminates the therapeutic process. In addition to the inherent inability of replication-incompetent viruses to spread beyond the initial cells infected, some of the disadvantages of these systems are the variability in transduction rates, the need for implantation of producer cells, and the possibility of insertional mutagenesis causing secondary tumors (*e.g.*, retroviruses). The second major approach is to use an engineered HSV with attenuated neurovirulence but which remains cytotoxic to tumor cells. In some instances, investigators have attempted to achieve this by creating HSV mutants deficient in one of the genes coding for enzymes needed for nucleic acid metabolism and replication in nondividing cells, *i.e.*, *tk* ($tk^-$) or ribonucleotide reductase ($rr^-$). Without these genes, the virus is unable to replicate unless the cell that it infects supplies the deficient enzymes, which are present normally only in dividing cells. Thus, viral replication is able to proceed in dividing cells and causes cell death. Unlike the above method of using a vector carrying the HSV-*tk* gene, this method allows the virus to spread to other proliferating tumor cells (18).

In addition to being replication-competent, these HSV mutants have the added advantage of not integrating into the host genome, eliminating the possibility of insertional mutagenesis. However, viral encephalitis is a potential major side effect of the use of HSV $tk^-$ (9). The virus is resistant to pyrimidine-analogue antiviral drugs (*e.g.*,

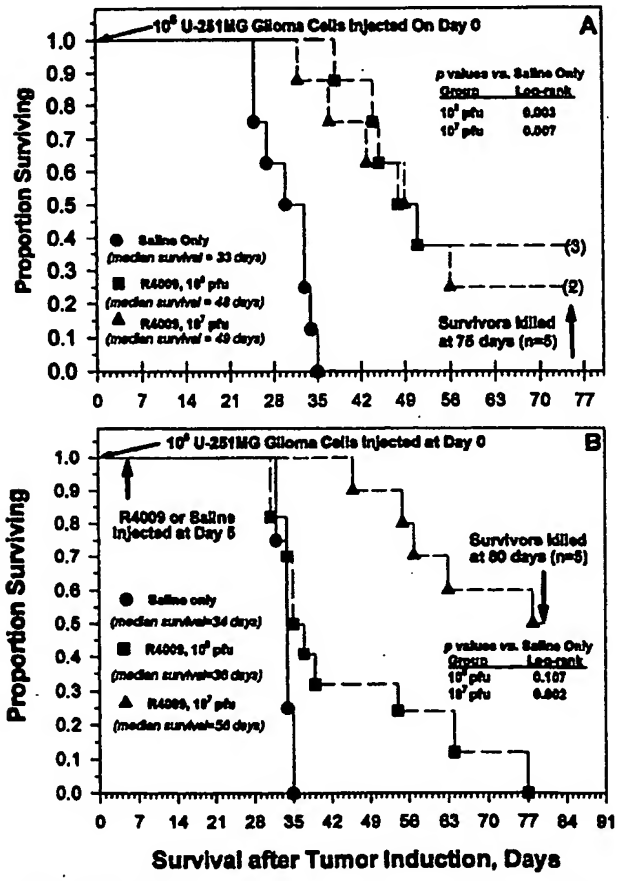


Fig. 6. Kaplan-Meier survival plots were constructed for *scid* mice injected with 5 μl of $10^6$ U-251MG human glioma cells intracranially. *A*, glioma cells were admixed prior to injection with $1 \times 10^6$ or $10 \times 10^6$ PFUs of HSV R4009. Long-term survivors were killed as indicated. *B*, glioma cells were administered intracranially, and 5 days later, mice were randomized into groups of 10 and either $1 \times 10^6$ or $10 \times 10^6$ PFUs of HSV R4009 were injected intratumorally in 5 μl.

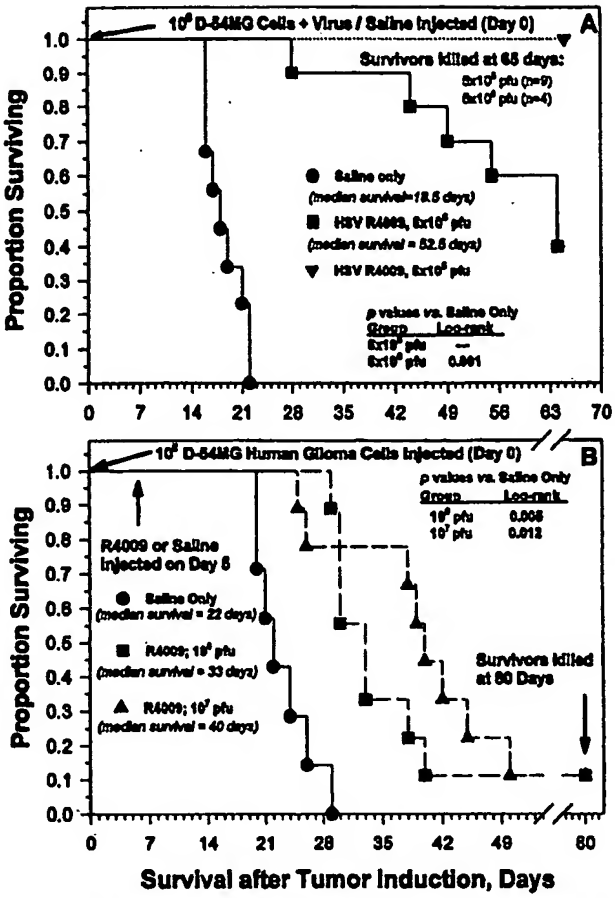


Fig. 7. Kaplan-Meier survival plots were constructed for *scid* mice injected intracranially with $10^6$ D54MG human glioma cells in 5 μl. *A*, glioma cells were admixed with $0.5 \times 10^6$ or $5 \times 10^6$ PFUs of HSV R4009 and then inoculated. Long-term survivors were killed as indicated. *B*, glioma cells were inoculated intracranially; 5 days later, mice were randomized into groups of 10 and either $1 \times 10^6$ or $10 \times 10^6$ PFUs of HSV R4009 were injected intratumorally in 5 μl.

ACV) but retains sensitivity to vidarabine and foscarnet (19). The $rr^-$ mutant retains the *tk* gene and is actually hypersensitive to GCV, which may help overcome this problem (20).

The $\gamma_1 34.5^-$ HSV mutants have not caused encephalitis in any treated animals with experimental brain tumors or in naive normal or *scid* mice at doses up to $10^7$ PFUs. R4009 has an $LD_{50} > 10^7$ PFUs, and the $LD_{50}$ of R3616 is $> 1.2 \times 10^6$ in immunocompetent BALB/c mice (21). In addition, these HSV mutants retain susceptibility to ACV (9). When U-87MG human glioma cells were xenografted intracranially in nude mice, median survival was extended from 42 days in control animals to 71 days in animals treated 10 days later with $10^5$ PFU R3616, with ~18% becoming long-term survivors (>120 days; Ref. 9). In our earlier studies of *scid* mice with semisyngeneic intracerebral grafts of MT539MG mouse glioma cells, median survival was increased by 10 days over controls with $10^6$ PFUs of R3616 or by 8 days with R4009 administered simultaneously and was increased by 9 days if R4009 was injected 72–96 h after the tumor cells (10). It was also found that R4009 was more efficient than R3616 in killing MT539MG cells in *In vitro* alamarBlue assays.[5] However, none of the mice bearing murine MT539MG gliomas were long-term survivors.

[5] Unpublished data.

Our present study showed that five different $\gamma_1 34.5^-$ HSVs were capable of killing a variety of human malignant glial cell types in culture; all of the tumor cell lines experienced a 50% reduction in dye conversion ($TD_{50}$) and, presumably, in cell viability. Furthermore, HSV cytotoxicity occurred following exposure to much lower quantities of genetically engineered HSV than observed for cultured normal human astrocytes. From these data, it would appear that some of the genetically engineered HSV viruses could be given in doses that would be effective against tumor cells without harming astrocytes, the other major cell type in the brain capable of being stimulated to divide. This margin of safety would be largest with R3616, which had no measurable toxicity to either culture of normal human astrocytes. However, R3616 was also the least potent virus against tumor cells, which may result in inadequate treatment in human populations. Our data also suggest, when comparing astrocyte susceptibility at 3 and 6 days, that the cytotoxic effects of HSV mutants toward astrocytes became more pronounced with time due, presumably, to a slower rate of spread of replicating virus. This may be related to the slower rate of astrocyte proliferation *in vitro* as compared to the rapid proliferation of malignant cells. The persistence of low levels of $\gamma_1 34.5^-$ HSV in brains of mice cured of intracranial glioma xenografts would suggest that replicating (gliotic) astrocytes may provide a reservoir for viral replication in an otherwise mitotically quiescent tissue. Persistent HSV infection in normal brain is clearly of concern. However, because $\gamma_1 34.5^-$ viruses retain the *tk* gene, they remain susceptible to GCV. Theoretically, GCV could be administered to end treatment after a significant oncolytic effect had been achieved but before significant toxicity to normal brain parenchyma occurs. We have demonstrated that systemic administration of GCV coincidentally with intratumoral R4009 therapy for intracranial gliomas in *scid* mice abrogated the protective effect of $\gamma_1 34.5^-$ HSV therapy (10). Clearly, $\gamma_1 34.5^-$ mutants offer clear advantages over other viral-based therapies and should be pursued as a potentially effective adjunctive treatment for lethal human brain tumors.

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